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Minireview

Developing microbe–plant interactions for applications in plant-growth promotion and disease control, production of useful compounds, remediation and carbon sequestration

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Summary

Interactions between plants and microbes are an integral part of our terrestrial ecosystem. Microbe–plant interactions are being applied in many areas. In this review, we present recent reports of applications in the areas of plant-growth promotion, biocontrol, bioactive compound and biomaterial production, remediation and carbon sequestration. Challenges, limitations and future outlook for each field are discussed.

Introduction

Interactions between plants and microbes are an integral part of our terrestrial ecosystem. There are several types of plant–microbe interactions: competition, commensalism, mutualism and parasitism. The more common interactions are commensalism or mutualism, where either one or both species benefit from the relationship respectively (Campbell, 1995). There are several excellent reviews reporting current research on lifestyles and molecular interactions of plant-associated bacteria (Sorensen and Sessitsch, 2007), rhizosphere interactions (Singh *et al.*, 2004a), plant responses to bacterial quorum-sensing (QS) signals (Bauer and Mathesius, 2004), endophyte applications (Ryan *et al.*, 2008), and rhizosphere bacteria responses to transgenic plants (Fillion, 2008).

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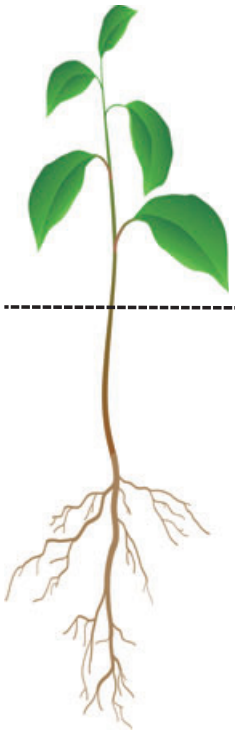
Examination of these interactions helps us to understand natural phenomena that affect our daily lives and could lead to applications resulting in sustainable resources, less impact on the environment, cleanup of pollution and influence on atmospheric gases on a global scale. Advantages of using these interactions for biotechnological applications are many-fold. The use of naturally existing plant-microbe symbiosis for plant growth and biocontrol reduces synthetic fertilizer and pesticide treatments leading to cost-effectiveness and less impact by nutrients (Boddey *et al.*, 2003) and pesticides (Whipps and Gerhardson, 2007; Elmer and Reglinski, 2006) on surrounding fauna and flora. The production of useful compounds with pharmaceutical and industrial relevance using plant–bacteria symbiosis is energy efficient (Wu *et al.*, 2007; Del Giudice *et al.*, 2008) and diminishes the need to add expensive precursors and catalysts. Remediation through conventional method, such as excavate and treat, is expensive and labour intensive. Conversely, plant–microbial remediation strategies can be less intrusive and much more economical (Anderson *et al.*, 1993). Carbon sequestration through plant–rhizosphere processes is a potentially sustainable method to lowering atmospheric carbon (Kumar *et al.*, 2006). This review focuses on recent progress in the fields of plant-growth promotion, plant disease control, production of bioactive compounds and biomaterials, remediation of contaminated sites, and carbon sequestration. The potential of applying these new developments is discussed. Figure 1 summarizes applications resulting from microbe–shoot and microbe–root interactions and techniques used. Table 1 is a glossary of the techniques mentioned in this review.

Plant-growth promotion

Plant–microbe interactions have been utilized to improve plant growth for the production of food, fibre, biofuels and key metabolites. The mutualistic interaction can be beneficial in directly providing nutrients to the plant (biofertilizer) or increasing the availability of compounds such as



Applications	Techniques	References
Plant Growth Promotion – N-fixing endophytes	Isotope analysis using MS, GFP labeled *Klebsellia pneumoniae* 342 (Kp342), genetic manipulation of Kp342, RT-PCR	Iniguez et al., 2004; James et al., 2002; You et al., 2005.
Biocontrol -*Pseudomonas* suppression of *Venturia inaequalis*	CCD camera, CSLM, RT-PCR, cDNA libraries, QTOF-MS	Gau et al., 2002; Singh et al., 2004; Degenhardt et al., 2005; Kurkcuoglu et al., 2007
Biocontrol- resistance to *Xanthomonas* sp. and *Xylella fastidiosa*	Recombinant *E. coli* and *Pseudomonas* spp.	Newman et al., 2008
Remediation – Toluene and BTEX	Recombinant *Burkholderia cepacia*, BOX-PCR genomic DNA fingerprinting, sequencing, GC-MS	Barac et al., 2004; Taghavi et al., 2005; Moore et al., 2005
Plant Growth Promotion- phytohormone	Ethylene measurement using GC	Mayak et al., 2004a; 2004b
Bioactive compounds production – Tashinone	Hairy roots tissue culture, HPLC	Wu et al., 2007
Bioactive compounds production – Rhizoxin	PCR, HPLC-MS, laser microscopy, ESI-MS, NMR, transcmission electron microscopy	Partida-Martinez and Hertweck, 2005
Bioactive compounds production – Essential oil	Box-PCR genomic DNA fingerprinting, GC-MS, RT-PCR, CSLM and TEM	Del Giudice et al., 2008
Remediation – Lindane	PCR, sequencing	Böltner et al., 2008
Remediation – PCB	Transgenic Arabidopsis and *P. putida* mutant, reverse-phase HPLC, ESI-MS, confocal microscopy	Narasimhan et al., 2003
Remediation – 4CNB	GFP-labeled *Comamonas* sp., confocal microscopy, quantitative-competitive PCR, PCR-DGGE	Liu et al., 2007
Remediation – Cadmium	Recombinant *P. putida*	Wu et al., 2006
Remediation – Cadmium and TCE	Recombinant *Pseudomonas* and *Rhizobium* strains, GC	Lee et al., 2006
Remediation – Cadmiun and zinc	DNA extraction and partial 16S DNA sequencing	Kuffner et al., 2008
Remediation – PCB and arsenic	Recombinant *P. fluorescens*, PCR	Ryan et al., 2007
Remediation – PCB	Recombinant *P. fluorescens*, CSLM, HPLC, PCR, sequencing	Villacieros et al., 2005; Leigh et al., 2006
Carbon sequestration	PLFA, Stable isotope probing	Bailey et al., 2002; Heinemeyer et al., 2007; Verburg et al., 2004; Bazot et al., 2006; Leake et al., 2006; Bremer et al., 2008; Pascal et al., 2006; Ingram et al., 2008

Fig. 1. Areas of symbiotic relationships (shoot and root) applied towards biotechnological applications and techniques used. The line separates interactions located in the shoot and root.

iron or phosphate. Free-living plant growth-promoting bacteria also produce compounds that directly affect plant metabolism or modulate phytohormone production or degradation. The phytohormones: auxins, cytokinins, gibberellic acid (GA_3), abscisic acid and ethylene are signalling molecules essential for growth which mediate a range of developmental processes in plants. Recent studies on each of these areas are presented in the following section.

Biofertilizers

As chemical fertilizers are costly to both the agricultural businesses and the environment, development of biofertilizers is an important and exciting area. Symbiotic relationships established between plants and bacteria such as rhizobium and some actinomycetes (e.g. *Frankia*) provide most of the nitrogen (N) available to legumes and actinorhizal plant species. A number of studies attempted to develop new association between non-legume plants and N-fixing bacteria; however, these attempts were mostly unsuccessful (Preininger and Gyurjan, 2001). The discovery of endophytic diazotrophs that provided reduced N to the non-legume plant species represents an alternative way of exploiting plant–microbe interaction for N nutrition. Brazilian sugarcane plants harbouring N-fixing endophytes were grown for many years with low fertilizer inputs, and showed no symptoms of N deficiencies (Boddey *et al.*, 2003). Other non-legumin plant species were also shown to benefit from association with diazotrophic endophytes, such as wheat (*Triticum aestivum*; Iniguez *et al.*, 2004) and rice (*Oryza sativa*; James *et al.*, 2002). Iniguez and colleagues (2004) generated a *nifH* mutant from *Klebsiella pneumoniae* and compared this to the wild-type strain in order to demonstrate that the wild-type *K. pneumoniae* strain provided N to the wheat plant thus alleviating N deficiency. The wheat plants were supplemented with stable N-isotope labelled nutrients. Mass spectrometry was used to show that less ^{15}N was present in wheat plants inoculated with the wild-type *K.*

Table 1. Glossary of techniques used to study plant–microbe interactions.

Techniques	Descriptions
Nucleic acid amplification and fingerprinting	DNA is amplified by polymerase chain reaction (PCR) while cDNA is synthesized from the extracted RNA. The amplicons can be analysed with fingerprinting techniques such as denaturing gradient gel electrophoresis, terminal restriction fragment analysis, amplified rDNA restriction analysis and BOX-PCR.
Real-time PCR (RT-PCR)	RT-PCR is based on the monitoring of PCR reaction using fluorescent reporter molecules. Particularly, analysis of the exponential curve of the PCR reaction allows the determination of the amount of starting material. Different detections methods have been developed such as SYBR green I, a fluorescent dye binding double-stranded DNA, and Taqman probes.
Sequencing	Determination of nucleotides sequence in single-stranded DNA or cDNA using Sanger or Pyrosequencing methods.
Chromatography	Chromatography allows for separation and isolation of chemical mixture by suspending compounds in solvent and separating mobile fraction from stationary fraction through a column. The proportions of the mixture provide quantitative information, and the separated fractions can be used for further analysis. Several types of chromatography have been described: thin layer chromatography, liquid chromatography, gas chromatography, high-performance liquid chromatography, ultra high-pressure liquid chromatography.
Mass spectrometry (MS)	MS identifies the chemical composition of molecules based on the mass-charge ratio of charged particles. Stable Isotope Probing with MS is being used to track a stable isotope atom from a particular plant substrate into microbial cells *in situ*. Electron-spray-ionization-MS is a technique for measuring chemical speciation in root exudates metabolomics. Quadrupole time-of-flight hybrid MS consists of both analysers resulting in high sensitivity in product ion scanning.
Phospholipid fatty acid (PLFA)	PLFA identifies phospholipids in cellular membranes that are esterified to fatty acids. GC is used to identify the fatty acids, and information regarding microbial biomass, metabolic status and community composition can be obtained.
Microscopy	The use of microscopy for visualization of plant-associated microbes provides useful spatial information that can be used to determine the functional relationships. Confocal laser scanning microscopy is used to obtain high-resolution optical sectioning images of fluorescent samples. Transmission electron microscopy bombards ultra-thin specimens with electrons in order to obtain images at higher resolution than light microscopes.
Fourier transform infrared spectroscopy (FTIR)	FTIR generates an infrared absorption spectrum specific for a type of chemical bond thus allowing for the identification of molecules.
Genetic modifications	Manipulating specific genes by both genetic engineering and mutation is used to validate previous observations regarding gene functions. Transgenic plants and recombinant microbes have been used.
Nuclear magnetic resonance	The technique measures physical resonance and is used to determine the structure of the chemical.

pneumoniae strain. As these plants obtained a higher percentage of their N from non-isotopically labelled atmospheric N_2, the majority of the plant nitrogen was derived from the wild-type strain. In a different study, the transcript abundance of the *nifH* gene was measured by RT-PCR in endophytic *Herbaspirillum* sp. during colonization of internal regions of wild rice plants (You *et al.*, 2005). Interestingly, they showed higher *nifH* transcript abundance during the light period compared with the dark period. Given the tight relationship between N-fixing activity and *nifH* gene transcription, the high-level of *nifH* mRNA during the light period indicated that the endophytes produced higher level of reduced N at a time when they could benefit most from photosynthate production. This study described a promising avenue for non-legume plants to obtain N from the reduction of atmospheric N_2. It remains to be seen whether similar results are achievable with soil grown plants or in the field where plants may be less limited for growth than in sand-filled pots. In addition, competition from other bacteria may prevent colonization by endophytic diazotroph.

Plant genetic contribution to this symbiosis is an important area of research leading to selection of agriculturally important plant genotypes with increased capacity to establish symbiosis for nutrient acquisition. Several studies have begun to pave the way in this area. The identification of genetic loci underlying complex traits in *Lotus* spp. and *Medicago* led to the sequencing of specific genes (Stougaard, 2001; Salvi and Tuberosa, 2005) essential for the establishment of root symbiosis. Kistner and colleagues (2005) characterized seven mutants of *Lotus japonicus* that were impaired in nodulation and were also defective in arbuscular mycorrhiza formation. Kanamori and colleagues (2006) used a F2 mapping population established by crossing the *Lotus japonicus* mutant and wild type for positional cloning of a gene essential for symbiosis that encoded a plant nucleoporin gene required for Ca^{2+} spiking occurring after contact between *Nod*-factor molecules and root hair cells. The combination of genetic and genomic analysis resulted in a deeper understanding of the plant genetic contribution to the establishment of symbiosis with rhizosphere bacteria. One drawback of this

approach is that it may take many years, from the identification of the genetic basis for complex traits to the development of new cultivars with enhanced capacity for establishing and maintaining symbiosis.

Phytohormone modulation

Rhizosphere bacteria and phyllosphere-colonizing epiphytes have been shown to produce a range of plant growth-stimulating phytohormones. A recent study by Boiero and colleagues (2007) evaluated the phytohormone synthesis of three commercially available strains of *Bradyrhizobium japonicum* grown in pure culture. They showed that the three strains have differential capability to produce the five major phytohormones: auxins, cytokinins, gibberellic acid (GA_3), abscisic acid and ethylene. This is important to consider as each individual and combinations of phytohormones may have different impact on plant growth.

The auxin compound indole-3-acetic acid (IAA) can be generated in bacteria through different biosynthetic pathways (Spaepen *et al*., 2007). Saravanan and colleagues (2008) reviewed the roles in plant growth promotion of two N-fixing organisms, *Gluconacetobacter diazotrophicus* and an *Acetobacteraceae* strain. In particular they discussed additional roles for *G. diazotrophicus,* including the production of plant hormones such as IAA, and gibberellins. Idris and colleagues (2007) showed for the first time that the Gram-positive bacterium *Bacillus amyloliquefaciens* produced and secreted significant amounts of IAA. They also showed the positive effect of IAA on the growth of *Lemna minor*. Patten and Glick (2002) demonstrated the direct positive effect of IAA produced by *Pseudomonas putida* through the indolepyruvic acid pathway, on root development. Roots from Canola seeds treated with the wild-type *P. putida* were longer than that of seeds treated with an IAA-deficient mutant. However, the bacterial production of IAA may not always be beneficial for plant as it is involved in pathogenesis and that high concentration of IAA can also inhibit root cell growth (Spaepen *et al*., 2007 for review).

Ethylene has many physiological effects on plant growth, development and modulation of responses to biotic and abiotic stresses. Bacteria such as *Pseudomonas* spp., *Burkholderia caryophylli*, *Achromobacter piechaudii* were shown to lower the endogenous ethylene level *in planta* by producing a degradative enzyme 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase (Mayak *et al*., 2004a,b; Shaharoona *et al*., 2007). The effects of ACC deaminase-producing rhizobacteria on plants included increased root growth, and improved tolerance of salt and water stress (Mayak *et al*., 2004a,b). These effects were noted in axenic conditions and more recently in field conditions. Inoculation of wheat (*Triticum aestivum* L.) plants with *Pseudomonas* spp. and *B. caryophylli* improved grain and straw yield by as much as 43% and 44% respectively (Shaharoona *et al*., 2007). However, no measurements of ethylene concentration were carried out on plants in pots or in the field to show direct correlation of ACC-deaminase production and ethylene degradation. It is therefore possible that other bacteria-associated effects might have improved wheat plant growth.

Cytokinins constitute a group of plant hormones that promote cell division in conjunction with auxin and are known to induce stomata-opening. Rhizosphere bacteria and fungi associated with plants were shown to produce cytokinins. Arkhipova and colleagues (2005) studied the production of different cytokinins by *Bacillus subtilis* and showed improved growth for lettuce plants (*Lactuca sativa* L.) after inoculation with cytokinin-producing bacteria. This was also the case when plants were grown under water stress (Arkhipova *et al*., 2007). The authors mainly considered the production of cytokinins by *B. subtilis* as mechanism of plant production. It would be interesting to investigate possible alternative mechanism by which this species is able to promote plant growth. Lettuce plants were grown in sand and the study did not address whether similar results could be obtained in the field where competition from other rhizosphere bacteria might prevent *B. subtilis* growth and where bacterial production of other phytohormones might interfere with the effect of cytokinins.

The modulation of phytohormone by bacteria that lead to disease resistance is addressed in the plant disease control section.

Future outlook

Plant growth-promoting bacteria have been shown to positively impact plant performance through different mechanisms. For future development of commercial inoculant, it is therefore important to consider all potential metabolic activity of the phytohormone-producing bacteria. In addition, components of the inoculant growth media could be transformed into metabolites (phytohormones) that could impact plant growth in the early stages of plant development (Boiero *et al*., 2007). For successful application of plant-growth promotion using bacterial inoculant, many aspects of the plant environment have to be considered in the field. In particular, it is important that the inoculant be beneficial to the crop, do not improve the growth of the weedy species and do not render the plants more susceptible to biotic and abiotic stresses. Bacterial colonization efficiency is also critical for successful inoculation. Further development of plant growth-promoting bacteria could benefit from selection, through breeding programmes of plant genotypes that respond better to the plant growth-promoting bacteria.

Plant disease control

The efficiency of biocontrol agents for plant disease has been demonstrated and some are commercially available (Whipps, 2004; Elmer and Reglinski, 2006; Whipps and Gerhardson, 2007). General mechanisms of action for plant pathogen control include competition for nutrients and space at the infection site, antibiosis, parasitism, production of cell wall-degrading enzymes, induced resistance in the plant, and manipulation of bacterial signalling molecules. It is likely that several mechanisms of action are at work in many biocontrol agents. Compant and colleagues (2005) reviews the mechanisms of plant disease biocontrol by plant growth-promoting bacteria. Here, we discuss two mechanisms of disease control: induced resistance and manipulation of signalling molecules.

Induced resistance

Interactions with bacteria can induce two types of plant defence responses that help protect against further infection. Systemic acquired resistance (SAR) is a specific response that triggers both a local increase in phytohormone accumulation and the formation of phloem mobile signal. Non-pathogenic free-living rhizosphere bacteria and endophytes can trigger the second type of plant defence called induced systemic resistance (ISR). A major distinction between SAR and ISR is the involvement of salicylic acid, with ISR being activated via an salicylic acid-independent pathway.

One example of plant disease control is the use of *Pseudomonas* strains with biocontrol activity to induce resistance in apple (*Malus domestica*) against the pathogenic fungus *Venturia inaequalis*, which causes apple scab. The movement of *Pseudomonas* on apple leaves was studied in order to understand its antagonistic interactions against *V. inaequalis* (Gau *et al*., 2002). Using confocal laser scanning microscopy, it was demonstrated that *Pseudomonas fluorescens* Bk3 localized near stomatal openings. The *P. fluorescens* Bk3 traversed the cuticle through secretion of cutinases, and acquired nutrient from fluid isolated from the apoplastic space. Also, isolated leaf cuticles stimulated bacterial extracellular proteins (Singh *et al*., 2004b). In order to understand the interaction between the *V. inaequalis* and apple tree, cDNA libraries using suppression subtractive hybridization were constructed for the resistant and susceptible cultivars (Degenhardt *et al*., 2005). Many plant defence-related transcripts such as those encoding for β-1,3-glucanase, cystein protease inhibitor and metallothioneins were at higher levels in the resistant cultivar. On the other hand, more RuBisCo transcripts were found in the susceptible cultivar than in the resistant one. Similar proteins were expressed in apple trees treated with the biocontrol strain *P. fluorescens* Bk3 and *V. inaequalis* (Kurkcuoglu *et al*., 2007). The presence of *P. fluorescens* Bk3 was demonstrated to elevate the defence mechanisms in the apple trees and could serve as effective biocontrol strategy. The authors implied that low levels of RuBisCo and high levels of metallothioneins resembled that of 'old leaves' and were 'unattractive' to *V. inaequalis* (Degenhardt *et al*., 2005). The authors did not investigate whether *P. fluorescens* Bk3 induced SAR or ISR type of plant resistance.

Manipulation of bacterial signalling molecules

Some bacteria rely on signalling molecules for the development of pathogenesis. Other microbes living in the same environment may degrade these signalling molecules. Recombinant biocontrol strains producing *carAB*, genes required for degradation of a fatty acid signalling molecule, were shown to reduce virulence caused by *Xanthomonas* sp. and *Xylella fastidiosa* (Newman *et al*., 2008). Secondary metabolites produced by plants were shown to mimic or inhibit QS molecules. *Medicago truncatula* was shown to produce more than a dozen compounds that stimulated or inhibited QS (Gao *et al*., 2003). Plants have been manipulated to produce molecules that mimic or block QS signal, and enzymes that degrade QS molecules or QS strategy. Scott and colleagues (2006) engineered tobacco plants to synthesize acyl-homoserine-lactones (AHL) in the chloroplast and demonstrated that AHL was transported and secreted on the phyllosphere and in the rhizosphere. Pretreatment of potato slices with *Bacillus thuriensis* resulted in reduced maceration from *Erwinia carotovora* virulence (Dong *et al*., 2004). The authors showed that the decreased pathogenesis involved the disruption of *E. carotovora* QS by deterioration of QS molecules with AHL-lactonase. Pretreatment of potato slices with *B. thuriensis* lacking the ability to produce AHL-lactonase did not reduce maceration. Manipulating plants to express AHL-lactonase has shown positive effect in protecting plants against pathogens (Zhang, 2003 for review). Recently, a gene called *qsdA* (for *q*uorum-sensing *s*ignal *d*egradation) from *Rhodococcus erythropolis,* encoding a new type of AHL-lactonase has been characterized. The gene was able to confer quorum-quenching capacity to *P. fluorescens* that led to enhanced protection of potato tuber against the soft rot pathogen *Pectobaterium carotovorum* (Uroz *et al*., 2008).

Future outlook

Elucidating the mechanism by which bacteria elicit ISR is important for the development of commercial biocontrol agent. Flagellin, siderophores, lipopolysaccharides and

more recently volatile organic compounds have been proposed as determinants that trigger ISR (Compant *et al.*, 2005 for review). Recent studies have also suggested that exopolysaccharides produced by *Bukholderia gladioli* are able to elicit ISR in cucumber plants (Park *et al.*, 2008). A better understanding of bacterial elicitor of ISR may eventually lead to the development of disease resistance strategies for crop protection.

Despite their potential use as biocontrol agent, bacterial inoculant are not widely used in agricultural setting. While their success has been demonstrated in some field trials, they are not consistently efficient in diverse field conditions (Mark *et al.*, 2006). One characteristic that is paramount to the success of bacterial inoculant is a high efficiency in colonizing the rhizosphere. The disruption of QS either by plants or bacteria have potential use in plant disease protection. However, it has relied on the genetic manipulation of plants or microbes thus far. The release of these genetically modified organisms might lead to potential risk to the crop supplies. Furthermore, it has been shown that plants can detect AHL and take this cue to enhance their defence against pathogens (Mathesius *et al.*, 2003). Therefore, the artificial quenching of AHL molecules could also interfere with natural adaptation of the plant's defence system. Overall, it seems that while quorum quenching may delay the onset of pathogenesis it does not prevent diseases.

Production of bioactive compounds and biomaterials

Secondary metabolites produced by plants constitute a major source of bioactive compounds that can be used as therapeutic agents or for biomaterial production. Recent studies have shown that plant–microbial interaction can be exploited to enhance the production of important secondary metabolites. The infection of plants by *Agrobacterium rhizogenes* produced hairy roots that showed high growth rate and branching, and were used for production of useful compounds (Guillon *et al.*, 2006 for review). Plant–bacteria interaction can be used to improve production of secondary metabolites by hairy roots. For example, Wu and colleagues (2007) established co-cultures of *Salvia miltiorrhiza* bunge hairy roots with *Bacillus* sp. and demonstrated enhanced production of tashinone. Tashinone is a bioactive compound in *S. miltiorrhiza* bunge root, used in Chinese medicine, for the treatment of menstrual disorders and cardiovascular diseases and prevention of inflammation. The hairy root culture is an exciting example of plant–microbe interaction that is now moving towards large-scale industrial application. Scaling up hairy roots culture for production of secondary metabolites may not be straightforward due to the complex features of hairy roots. Please refer to other reviews that discussed aspects of this process (Georgiev *et al.*, 2007; Srivastava and Srivastava, 2007).

Rhizobacteria was shown to induce the accumulation of sesquiterperne synthase transcripts (Del Giudice *et al.*, 2008). Sesquiterperne, used in cosmetics and perfumery, is one component of vetiver root essential oil synthesis. The authors showed that the root-associated bacteria metabolized the vetiver oil and produced additional compounds, which suggested that each distinct rhizobacteria community contributed to a signature composition of commercial vetiver oil.

For two decades, it was thought that the plant pathogenic fungus *Rhizopus microsporus* produced the antimitotic polyketide macrolide rhizoxin. Partida-Martinez and Hertweck (2005) uncovered that it was actually an endosymbiont, *Burkholderia* sp., of the fungus that produced rhizoxin. The authors demonstrated that in the absence of *Burkholderia*, no rhizoxin was produced in the fungal culture. Transmission electron microscopy demonstrated that *Burkholderia* sp. was localized in the fungal cytosol (Partida-Martinez *et al.*, 2007). Rhizoxin inhibits mitosis and leads to cell cycle arrest, and has potential as an antitumor drug. Further investigation elucidated rhizoxin derivative structures and obtained stable analogues by inhibition of a putative P-450 monooxygenase (Scherlach *et al.*, 2006).

Ryan and colleagues (2008) summarized applications for bacterial endophytes, including production of biomaterials such as poly-3-hydroxybutyrate and poly-3-hydroxyalkanoate. Catalán and colleagues (2007) showed that diazotrophic endophyte *Herbaspirillum seropedicae* could accumulate 36% of its biomass as poly-3-hydroxybutyrate and could constitute a cost-effective means for producing biomaterial.

Future outlook

The above example of three-way interaction between plant–bacterium–fungus provides a reason to step beyond pair-wise interactions and currently existing model systems (Douglas, 2008; Heath, 2008). Marra and colleagues (2006) examined the three-way interactions among bean plant (*Phaseolus vulgaris*), two pathogenic fungi and an antagonistic *Trichoderma* spp. and found that ISR was achieved. Focusing on interactions from a community perspective may reveal further useful applicable mechanisms.

Remediation

Researchers have explored the use of plant–microbe symbiosis for remediation of pollutants since the early 1990s (Anderson *et al.*, 1993; Brazil *et al.*, 1995). Investigations in this area have progressed from bench-scale

experiments towards addressing more *in planta* field remediation efficiencies in recent years. Current phyto- or rhizo-remediation systems targeting contaminants such as recalcitrant chlorinated compounds, volatile organic carbons and heavy metals are highlighted here.

Recalcitrant-chlorinated compounds

Organic contaminants naturally biodegrade, except for chlorinated compounds such as polychlorinated biphenyl (PCB) and 4-chloronitrobenzene (4CNB), which tend to be persistent and recalcitrant in the environment. Recent reports on chlorinated compound degrading rhizosphere bacteria are presented. Natural PCB-degrader bacterial populations were cultured from several plant species growing in a contaminated site. High numbers of culturable PCB-degrader colonies were isolated from roots of Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) (Leigh *et al*., 2006). Most of the PCB-metabolizing bacteria are *Rhodococcus* sp. However, the isolates were first selected based on cultivability and morphology thus possibly leading to a biased representation of PCB-degrading bacterial communities. In addition, as the PCB-degradation capability of these strains was tested in liquid medium, the benefits of plant association was not addressed.

Narasimhan and colleagues (2003) investigated the role of plant secondary compounds in stimulating rhizobacteria growth and PCB removal efficiency. The authors identified that the *Arabidopsis* root exudates consisted mostly of phenylpropanoids, such as flavonoids, lignins and indole compounds. Wild type and mutant *Arabidopsis* lines over producing flavonoids sustained higher counts of flavonoid-utilizing *P. putida* strain than bacteria grown on *Arabidopsis* mutant not producing flavonoids. More interestingly, close to 90% of PCB was degraded in soil adhering to the roots indicating that direct contact with roots and the exudates resulted in bacteria growth and biodegradation enhancement. Genetically engineered microbes were also applied towards remediation. The PCB-degradation efficiency of a recombinant strain of *P. fluorescens* expressing a *bph* operon under the control of nodulation (*nod*) genes from *Sinorhizobium meliloti*, was examined (Villacieros *et al*., 2005). Resting cell PCB-degradation experiments indicated that the recombinant strain metabolized different cogeners of PCB more efficiently than the *Burkholderia* sp. strain LB400. However, no plant–bacteria potted experiments were performed to measure PCB degradation.

Böltner and colleagues (2008) enriched rhizosphere microbes and demonstrated that four *Sphingomonas* strains were capable of rhizoremediating hexachlorocyclohexane (Lindane). Potted experiments showed that 30% of lindane was removed with corn seedlings inoculated with the *Sphingomonas* strains. Whereas, in unplanted soil, sterile planted soil, and uninoculated unplanted soil, less than 3% of lindane was removed.

Liu and colleagues (2007) successfully inoculated a *gfp*-tagged strain of *Comamonas* sp. onto the roots of alfalfa as demonstrated by quantitative competitive-PCR and confocal laser scanning microscopy. The rhizosphere community shifted due to addition of 4CNB and the *Comamonas* strain was characterized using denaturing gradient gel electrophoresis. In outdoor potted experiments, the symbiosis enhanced phytotoxicity resistance, and removed 4CNB faster than the plant control without the strain in a 24-hour period. However, the control without the strain removed 60% of the 4CNB compared with 99% in experiment with strain inoculation. It seemed likely that if the experiment was extended beyond the 24 h measurements, the 4CNB removal might be similar between the control and the treatment.

Volatile organic compounds

Phytoremediation has been used as a treatment method to remove contaminants from groundwater. However, certain volatile organic compounds such as trichloroethylene (TCE) and BTEX (benzene, toluene, ethylbenzene, xylene) are released into the atmosphere through the plant's vascular system. The endophyte–plant interaction was used to degrade these volatile organic pollutants and minimize evapotranspiration (Barac *et al*., 2004; Taghavi *et al*., 2005; Moore *et al*., 2006). Barac and colleagues (2004) demonstrated that the genetically modified endophytic strain of *Burkholderia cepacia* together with yellow lupine (*Lupinus luteus L*.) reduced evapotranspiration of toluene by 50–70%, and reduced phytotoxicity. The same group inoculated poplar cuttings with two strains of *B. cepacia*, an endophyte and a soil isolate, expressing the toluene monooxygenase (*tom*), and showed significantly less toluene being transpired in the poplar inoculated with the endophytic *B. cepacia* (Taghavi *et al*., 2005). In addition, the authors indicated evidence for horizontal gene transfer of the recombinant plasmid encoding the toluene degradation pathway from the inoculant *B. cepacia* to the endogenous microbial community, and suggested that it would be possible to eliminate selection of an appropriate endophyte because the biodegradation gene will be transferred to the endogenous microorganisms. The authors touched on the issue of low horizontal transfer efficiency but did not elaborate on solutions for the lack of control over the time scale of the transfer and recipients of the genes. The authors also did not comment on the risks associated with the spread of exogenous genes into a new environment. However, the risk caused by a gene originally isolated from the environment, such as the *tom* gene, should be low compared with risks from synthetic genes.

Moore and colleagues (2006) isolated endophytic bacteria from two poplar varieties that were grown at a field trial site phytoremediating BTEX in the groundwater. There were differences in spatial compartmentalization of strain localization in the root, shoot and leaves suggesting there were species-specific and non-specific associations between bacteria and plants. However, the study did not examine the reasons for the strain localization and association with the plants and whether remediation efficiency was affected by bacterial community.

Metals

Heavy metal contamination is a persistent problem because the metals, unlike organic compounds, do not biodegrade. Several recent reviews address the role of plant growth-promoting rhizobacteria in metals remediation (Khan *et al*., 2009), and various plant–bacteria systems that have been applied towards remediation of metals contamination (Kamaludeen and Ramasamy, 2008). Removal of metals from soil or groundwater using rhizosphere bacteria and plants has been demonstrated recently. Willow (*Salix caprea*) seedlings inoculated with different strains of rhizosphere *Streptomyces* and *Agromyces* and the strains ability to produce IAA and siderophore was measured. No correlation was observed between IAA and siderophore production and metal accumulation. Only plants inoculated with *Agromyces terreus* exhibited reduced phytotoxicity due to cadmium and zinc. Metal accumulation in leaf biomass was measured (Kuffner *et al*., 2008). However, the data did not demonstrate a marked difference between the inoculated plants and the non-inoculated control. Perhaps, most of the phytoaccumulated metals were immobilized in the roots where most of the rhizosphere strains would be located. No root metal concentrations were measured.

Wu and colleagues (2006) utilized an engineered symbiosis between recombinant *P. putida* and sunflower plants (*Helianthus annuus*) for adsorption of cadmium. The recombinant *P. putida* expressed a synthetic phytochelatin, EC20, and exhibited inherent cadmium resistance. The inoculation of the *P. putida* strain enhanced plant growth and resulted in 40% more cadmium accumulation from hydroponic solutions than the non-inoculated control. No field study was conducted. Ryan and colleagues (2007) demonstrated that a recombinant strain of *P. fluorescens* F113rifPCB with arsenic resistance genes and PCB-degradation capabilities protected *M. sativa* when grown in soil supplemented with sodium arsenate. No arsenic removal or PCB-degradation efficiency were assessed with this plant–bacteria system. As most of the metals tend to accumulate in the top 20 cm layer of the soil (Li *et al*., 2009), grass species with high fine-root biomass providing large surface area for bacteria colonization and metal accumulation, in the top soil layer, would be ideal for remediation of metal contaminated sites. The plants serve as concentrators of metals, and further treatment of the plant biomass accumulated with metals would be required. Overall, the use of the plant–bacteria system would be more cost-effective than excavation of the soil contaminated with low levels of heavy metals.

Future outlook

The successful demonstrations of laboratory-scale phyto- or rhizo- remediation do not always translate into adequate removal of contaminants in field-scale. The reasons include heterogeneity of the field site, unpredictability of environmental conditions, inability to sustain bacterial population and the remediating microbes being outcompeted by endogenous organisms. There are several reasons that plant–bacteria interactions are advantageous when applied to remediation. The plant-associated bacteria population would be more competitive than the native soil microorganisms because plant exudates provide nutrients. Plants would act as natural pumps that draw contaminated soil pore water towards the plant-associated remediating bacteria. Rhizoremediation is an ideal strategy for cleanup of mixed-contaminants. Recombinant rhizosphere bacteria with specific genes targeting pollutants present at the site can be inoculated into plant roots. For example, Lee and colleagues (2006) engineered two strains of rhizobacteria with TCE-degradation capability, and surface expression of synthetic phytochelatins for improved heavy metal resistance, thus enhancing the rate of TCE degradation.

An excellent review by Gerhardt and colleagues (2009) addressed strategies to overcoming challenges of field application of phyto- or rhizo-remediation such as stressors to the microbes, complexity of the field conditions, regulatory acceptability and the use of genetically modified organisms. Research that looks at the impact of genetically modified organisms on native bacteria (de Cárcer *et al*., 2007a) and bacterial community changes post introduction of willow trees (*S. viminalis*) for rhizoremediation of PCB contamination (de Cárcer *et al*., 2007b) are starting to emerge and will elucidate questions regarding the impact on the native microfauna. Studies that compare remediation efficiencies of natural attenuation, bioaugmentation, phytoremediation and rhizoremediation, under field conditions (van Dillewijn *et al*., 2007), assess bacterial colonization (Molina *et al*., 2000; Watt *et al*., 2006) and activity (Wu *et al*., 2008) on plant roots, and measure allometric relationships of tree trunk size and various root parameters (Olson *et al*., 2003) provide invaluable information for determining field application options and evaluating contaminant removal. More extensive, long-term field comparisons of control plots with

treatment plots for remediation efficiency are necessary to validate laboratory observations and to gain public and regulatory agency confidence for this promising application.

Carbon sequestration

Another application of plant–microbe interaction drawing increasing attention is carbon (C) sequestration, where atmospheric C is deposited as plant root material, incorporated into the soil microorganisms and soil organic matter. It is hypothesized that increase in CO_2 leads to an increase in rhizodeposition, and wider C/N ratio thus retarding decomposition (Norby and Cotrufo, 1998). Several reviews are dedicated to presenting methods for measuring CO_2 fluxes in different soil compartments (Kuzyakov, 2006), mechanism of root carbon stabilization (Rasse *et al*., 2005), effects of elevated CO_2 on belowground carbon storage (Pendall *et al*., 2004) and carbon sequestration by roots (Kumar *et al*., 2006). This nascent field is still at the exploratory stage where most of the research is focusing on understanding the effects of elevated CO_2 levels on microbial community, belowground plant material production, and biomass decomposition, as well as land management practices and plant species on the long term potential of C rhizodeposition.

Effects of land management practices and fertilizer application

Several researchers explored the possibility of different land management practices to enhance C sequestration. Bailey and colleagues (2002) compared *ex situ* incubations of soil samples from five different ecosystems (desert, restored tallgrass prairie, Douglas fir forest, loblolly pine forest, and agricultural land). The restored prairie samples had the highest total soil carbon and also the highest fungal-to-bacterial activities (F : B). The authors asserted that increased F : B ratios correlated with higher amount of carbon stored in the soil. Also, that invasive land management decreased fungal biomass and thus the carbon stored in the soil. Soil samples were collected and CO_2 respiration experiments were conducted in a laboratory setting over a 6-h period, thus the measurements might not be representative of field conditions.

Heinemeyer and colleagues (2007) described contradicting results in their *in situ* study of the ability of Lodgepole pine associated mycorrhiza to store soil C over a period of 1 year, where the fungus was thought to return plant surplus C directly back to the atmosphere. Verburg and colleagues (2004) compared the net ecosystem carbon exchange between the atmosphere and two experimental non-native cheat grass (*Bromus tectorum*) varieties in a 2-year study in the Desert Research Institute's (Nevada, USA) Ecologically Controlled Enclosed Lysimeter Laboratories. They showed that fertilization increased C uptake initially; however, C loss through soil respiration was also enhanced.

Bazot and colleagues (2006) found similar results, at a grassland ecosystem of Free Air CO_2 Enrichment (FACE) in Eschikon, Switzerland, where increased N supply to the plants enhanced allocation of fixed C to the shoots and reduced belowground carbon allocation and rhizodeposition. The plots were enriched with CO_2 for 9 years in this study. At the same Swiss FACE facility, changes in microorganism structural diversity and function were also examined after 9 years of CO_2 enrichment in monocultures and mixed cultures of *Trifolium repens* L. cv. Milkanowa and *Lolium perenne* L. cv. Bastion with and without N treatments (Drissner *et al*., 2007). The authors concluded that increased atmospheric CO_2 stimulated microbial enzymatic activities and changed structural diversity. Subsequently, the increase in microbial activity led to higher mineralization rate of soil organic matter and thus would reduce the C sequestered in the soil. However, the soil C concentrations were not quantified in this study.

Long-term field studies

Long-term effects are essential for assessing the applicability of plant-rhizosphere C sequestration potential, and are investigated in the following field studies. Ingram and colleagues (2008) investigated microbial community changes to cattle grazing practices over a 10-year period at the High Plains Grasslands Research Station (Wyoming, USA). The authors found that even moderate grazing impacted microbial community structures and vegetation composition that could lead to loss of soil organic C to the atmosphere. In the heavily grazed area, there was a 30% loss of soil organic C and the measured N-mineralization rate was the lowest among the different grazing regimes.

A 5 year study of loblolly pine-associated fungi at a FACE site (North Carolina, USA) resulted in an increase in fungal biomass and a shift in distribution to deeper soils (Pritchard *et al*., 2008). Leake and colleagues (2006) presented a comprehensive study on C fluxes from plants to soil microbiota using $^{13}CO_2$ pulse-labelling on a Scottish upland grassland over a period of 4 years. It was found that there were two distinct pools of soil carbon: one with fast turnover and one with slow turnover time. Mycorrhizal mycelial system contributed to the pool with the fast turnover, by consuming approximately 9% of the fixed C, and returning much of it to the atmosphere within 16 h. Liming was found to increase shoot and root productivity. However, liming enhanced soil respiration and microbial biomass production, thus decreasing the amounts of C retained in the soil.

Bremer and colleagues (2008) compared soil organic C 6 and 12 years after a crop rotation study commenced

and found that the C sequestration of the different soil treatments did not increase after the first 6 years for the carbon-conserving practices. However, fertilized grass continued to gain soil organic C over the 12 year period. Niklaus and Falloon (2006) studied the capacity of calcareous grassland exposed to high levels of CO_2 for 6 years and found that the C sequestration potential was limited due to processes that were unaccounted for such as increased soil moisture due to reduced leaf conductance, soil disaggregation due to increased moisture and accelerated soil organic matter decomposition. A 30 year soil C projection modelling using field measurements was used to assessing affects of ambient and elevated CO_2 level (Sindhøj *et al.*, 2006). The results did not support the hypothesis that decreased litter quality due to increased CO_2 level would lead to lower decomposition rate (Norby and Cotrufo, 1998). The authors suggested that it would be more effective to reduce the rate of decomposition, rather than increase storage of plant litter C.

Future outlook

The studies presented in this review do not encompass all possible plant–microbe C sequestration research available. However, most of the studies discussed here indicate that optimal conditions for belowground C storage have not been found. There are many possible direct and indirect effects on the belowground C pools and processes resulting from elevated CO_2 and increased temperature (Pendall *et al.*, 2004). Microbial activity is affected by increased temperature leading to increased N availability and net primary production. There might be differences in C sequestration capacity for different plant species, and whether they are perennial or annual. The water content of the soil and N-limitation would affect microbial mineralization of organic soil C. The challenges may lie in improving measurement methods of C stored below ground and de-convoluting the myriads of confounding factors that could affect C and N cycling. In addition, the scale of the application required to have an impact on the atmospheric C level is unclear at this point. More long-term and standardized studies, under different environmental conditions, of belowground carbon fluxes, integrating models and measurements are needed. C sequestration through plant–microbe interaction is still in its exploratory phase. As more worldwide attention is drawn towards mitigating elevated atmospheric C level, hopefully more global collaborative interdisciplinary research efforts will be directed towards assessing the conditions required for successful application of plant–microbe C sequestration.

Conclusion

The biotechnological applications presented in this review are evident of a human–plant–microbe symbiosis where all three benefit from one another. Applications with plant–microbe interactions provide more economical and environmentally sound alternatives to conventional processes. Successful practice of these applications will depend on enhancements in the following areas. The sometimes confounding results in field applications might result from varying environmental conditions or species specificity and, thus, warrant comprehensive investigations of basic mechanisms and evaluation of responses. Knowledge of available techniques and continual improvement are important first steps to addressing the unknowns in this complex network. Research in microbe–plant interactions will benefit greatly from development of new techniques. A global approach in quantifying all possible changes is necessary, and is now feasible with the development of high-throughput techniques in genomics, metagenomics, transcriptomics, proteomics and metabolomics. These techniques produce a sizeable dataset and require sophisticated statistical analyses and modelling (Fiehn, 2002). The results from the global analyses would elucidate key pathways or genes. Genetic manipulation in plants and microbes altering metabolic pathways and genes is needed for validation. Most importantly, long-term field treatment efficacy and impacts associated with use of genetically modified organisms need to be assessed thoroughly (Fillion, 2008). Expansion of plant transgenic and microbial recombinant protocols would facilitate the validation process, as well as the development of new applications.

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